The Pigmentary Effector System. I.—Reaction of Frog's Melanophores to Pituitary Extracts.

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CONTENTS.

PAGE

1. Introduction 318
2. Specificity and Localisation of a Pituitary Melanophore Stimulant 320
3. Sensitiveness of the Melanophores to Pituitary Extracts 322
4. Relation of the Melanophore Stimulant to the other Pituitary Autocoids 323
5. Relation of the Melanophore Stimulant to Histamine 325
6. Mode of Action of the Melanophore Stimulant 326
7. Summary 328

1. *Introduction.*

The ability of certain organisms, including notably the Mollusca and lower Vertebrata, to respond to their surroundings by appropriate pigmentary changes has long been familiar to biologists; it has been recognised for more than half a century that a special type of effector organs (chromatophores, melanophores, etc.) are actively instrumental in producing such changes, and that, in Vertebrates at least, pigmentary response is partially controlled through the nerves by stimuli received from the organs of vision. During the past two decades it has been shown that the reactions of the pigment cells to stimuli simulate those of other effector organs, especially as regards their responses to certain internal secretions. That a fuller understanding of their properties might prove of service to practical aspects of physiology, as well as the key to a knowledge of "colour adaptation," was realised by Lister, who concludes his paper on the cutaneous pigmentary system of the Frog (1858) with the following comment: "The pigmentary system also promises to render good service in toxicological enquiry. Hitherto in experiments performed on animals with that object attention has been directed chiefly, if not exclusively, to the effects produced upon the actions of the nervous centres, the nerves, and muscles. In the pigment cells we have a form of tissue with entirely new functions, which, though apparently allied to the most recondite processes, yet produce very obvious effects. . . . Such experiments are so readily performed, and the effects produced are so obviously indicated by the changes in colour of the integument, that I venture to recommend this method of investigation to those who are occupied in studying the action of poisons. . . ."

A considerable literature, which has been reviewed too thoroughly and recently to merit an extensive epitome here, bears witness to the intricacy of the mechanism which underlies pigmental response in Fishes, Amphibia, and Reptiles; and very little work has been done on Molluscs in this connection. It can be safely asserted that as many distinct types of chromatophores are already known as there are different sorts of other effector organs whose properties have been studied. Hence it is obvious that, apart from the intrinsic interest presented by the phenomenon of colour change, a study of the properties of this series of effectors invites consideration in relation to a fuller understanding of the *rôle* played respectively by the character of the tissue and the nature of its nerve supply in the local action of drugs. The research, of which this preliminary communication contains an initial account, of the response of Amphibian melanophores to pituitary extracts, has been approached with both these objectives in view. For present purposes it will suffice to recapitulate briefly what has already been achieved in relation to the endocrine factors in pigmental response.

Earlier workers on the pigment effector mechanism concentrated their attention on the nervous control. Corona, however, showed as early as 1898 that a glandular extract could induce pigmental changes in the frog when he brought about contraction of the pigment cells with adrenalin. Lieben (1906) confirmed this conclusion, and a similar reaction of pigment cells to adrenalin has been demonstrated in other Amphibia and in Fishes by various workers. Contraction of the melanophores of frog tadpoles was found to follow pineal administration by McCurd and F. Allen (1917), whose results have been since confirmed by Huxley and Hogben (1921). The latter have also shown that melanophore expansion follows pituitary feeding in salamander larvæ, an observation which accords with the condition of general melanophore contraction seen to follow pituitary extirpation by Allen (1916), Smith (1917), Atwell (1919), and Swingle's (1921) experiments on grafting pituitaries, published while these experiments were in progress. These workers experimented on Amphibia; Spaeth (1917), on the other hand, found that the melanophores of isolated scales in the Teleost fish *Fundulus* contract in response to both pituitary and adrenal preparations.

Whatever may be true in the case of Fishes, this is certainly not the mode of response among Amphibia, as the present experiments are intended to indicate. Pituitary extracts injected into the frog produce a visible darkening, with complete and extreme expansion of the melanophores. The results recorded show that this is a specific reaction of extracts of the posterior lobe, is produced by a principle probably identical with the uterine stimulant, and is independent of the nerve-endings in the local effect which it evokes.

The normal reactions of melanophores to light in the frog depend on several factors besides light, temperature being of predominant significance. For the purpose of the experiments, it was necessary to use frogs in which the melanophores were fully contracted. Decerebrated animals were employed: the room temperature was uniformly 73° to 77° F. At this degree of warmth, the frogs placed on a white background under bright illumination show complete contraction of the melanophores, which condition persists after the animals have been decerebrated carefully, so as to avoid injury to the pituitary of the animal itself. Throughout this paper, the melanophores referred to are in all cases the *dermal* melanophores.

2. *Specificity and Localisation of the Pituitary Melanophore Stimulant.*

The experiments recorded in subsequent sections were based upon pituitary extracts prepared commercially for clinical purposes. Such preparations are made from the posterior lobe of the gland, which includes not only the infundibulum *sensu stricto*, *i.e.*, the pars nervosa, but, in addition, the pars intermedia, which is ontogenetically a hypophysial structure, though the term hypophysis is sometimes inaccurately applied to the whole gland or to the pars anterior (anterior lobe) alone. Hence the term infundibulin applied to such extracts is misleading. The following preliminary experiments indicate the existence of a specific melanophore stimulant in pituitary extracts and its localisation in the pars intermedia and nervosa:—

(i) An adult female rabbit was decapitated, and its pituitary gland, together with pieces of muscle, brain, ovary, pancreas, suprarenals and spleen removed at once. The tissues were severally weighed, ground up with sand, made up to 1 per cent. in Ringer, and left in the thermostat at 35° C. for 2 hours. At the end of 2 hours eight pairs of pale frogs were taken, being injected in pairs (0·5 c.c. per individual), with the extracts of tissues enumerated, another pair being injected with a 0·1 per cent. solution of pituitary, and an additional pair with an extract of putrid meat. The object of the latter injection was to control the possibility that the effects found to follow injections of commercial products were not due to traces of the physiologically active (pressor) substance known to occur in putrefying tissues. After half an hour, the pairs injected with 1 per cent. and 0·1 per cent. pituitary extracts had assumed a coal-black hue, while all the others remained pale: microscopic examination of the skin showed that this effect was due to the expansion of the melanophores in the former case.

(ii) The small size of the pituitary gland in the rabbit as well as the fact that the rabbit's pituitary is remarkably compact, having hardly any cleft between the two lobes (which are thus additionally difficult to separate), made it impossible to test the effect of extracts from different parts of the gland in the foregoing experiment. In the case of the ox pituitary the demarcation between the three parts is very striking. It was possible to secure ox pituitaries from the slaughter house within little more than an hour of killing, and to dissect away portions of the pars nervosa, intermedia and anterior separately, for preparing extracts of the three divisions of the gland. Each portion was weighed, ground up with sand, and after extraction with Ringer at 35° C. for 2 hours made up to a 0·1 per cent. and 0·02 per cent. solution. A pair of pale frogs was injected with each of the six solutions (0·5 c.c. per individual). After twenty minutes the four frogs injected with anterior lobe extract showed no darkening. The pair injected with weak (0·02 per cent.) pars nervosa extract likewise displayed no darkening, whereas both frogs which had been injected with 0·1 per cent. pars nervosa extract and all four animals injected with the pars intermedia preparation (0·1 per cent. and 0·02 per cent.) showed intense darkening of the skin. Microscopic examination showed that the three pairs injected respectively with pars anterior, strong and weak extracts, and pars nervosa weak extract, had the melanophores in the contracted condition, while the remaining six, those injected with pars intermedia and pars nervosa strong extract, displayed a state of general melanophore expansion of the characteristic type.

From these experiments it is clear that the pituitary gland contains a specific principle which is capable of inducing an extreme type of melanophore expansion in the frog, and that the production of this substance is located in the posterior lobe. It will be noted that the extract prepared from the pars intermedia was in the last experiment more potent than that prepared from the pars nervosa in corresponding amounts of the fresh glandular substance. The fact that workers like Swingle, who record the effect of grafting experiments, claim for the pars intermedia an exclusive *rôle* in the pigmental control of the pituitary gland, does not necessarily conflict with this result, for it is on histological grounds unlikely that the pars nervosa actually secretes the autocoid which produces melanophore expansion. What is most likely, as has been suggested by other writers, is that the pars intermedia secretion rapidly diffuses into the nervosa, in which case the term infundibulin applied to such extracts is not merely confusing but positively incorrect. The changes which follow injection of pituitary extracts in the frog confirm the conclusion that the condition of pallor which results from pituitary removal in amphibian larvæ, as shown by Allen and others, is due to endocrine deficiency.

3. *Sensitiveness of the Melanophores to Pituitary Extracts.*

The following tests indicate the mode of response of frogs' melanophores to different concentrations of pituitary extracts. Owing to the extreme simplicity of the technique employed, it is not desirable to repeat the description where experiments have been repeated for confirmatory purposes. The extract employed was in all the remaining experiments Burroughs and Wellcome's liquid sterile posterior lobe extract ("infundin") in 0·5 c.c. tubes.

(1) Solutions 0·5 per cent, 0·05 per cent., 0·005 per cent., 0·0005 per cent. of the liquid extract in frog's Ringer were made up, 1 c.c. of each solution being injected into each of a pair of pale frogs; a fifth pair was injected with saline simultaneously as a control. At the conclusion of half an hour (by which time maximum reaction is attained with pituitary extracts), the two pairs injected respectively with 0·5 per cent. and 0·05 per cent., showed the striking pigmentary changes described in the last section; the remainder were still pale. On removing a piece of skin from the back of each frog, fixing in Bouin's fluid, dehydrating and mounting in balsam, it was seen that in the two pairs that showed visible darkening, the melanophores were so fully expanded that their processes appeared to form a continuous web, thus rendering the skin almost opaque. The other frogs showed complete contraction of the melanophores, except in the 0·005 per cent. pair, in which there was perhaps a very slight tendency towards a stellate condition of the melanophores. The visible darkening of the skin appears within 10 to 20 minutes, reaches its maximum in half an hour, and disappears within 3 hours from the time when injection takes place, as was shown by a further injection of the 0·5 per cent. solution into another pair of animals. Pieces of skin were also placed in the stronger pituitary solution to test its action on the isolated skin. So treated, they displayed on microscopic examination half an hour later, complete expansion of the melanophores, while other pieces placed in saline showed the melanophores contracted to fine points. It should be explained, however, that if the skin is subjected to a good deal of mechanical stimulation, as for example, after being cut into small strips 2 mm. square, the melanophores remain contracted and will not respond by expansion to pituitary extract.

(2) A second tube of infundin was used to make up solutions, 0·1 per cent., 0·075 per cent., 0·05 per cent., 0·025 per cent., 0·01 per cent. and 0·005 per cent. Six pairs of frogs were taken and injected (0·5 per cent. per individual)

severally with the above strengths. The first and fifth doses were, it will be noted, quantitatively equivalent to the second and third in the foregoing experiment. At the end of half an hour the first two pairs (0·1 per cent. and 0·075 per cent.) were intensely dark; the others showed but slight, if any, perceptible change. Microscopic examination of pieces of the skin showed that in the first two pairs the melanophores were expanded, whereas in the remainder they were contracted. It may be well to observe parenthetically in this connection, that the terms *contraction* and *expansion* are used in a purely descriptive sense throughout this paper, without prejudice to the controversial issue, to be dealt with subsequently it is hoped, whether the pigment cell as a whole contracts and expands, or whether on the other hand the appearance is due to the independent migration of the pigment granules.

The above experiments illustrate two significant aspects of the pituitary melanophore reaction: (*a*) there is a fairly definite end point; and (*b*) it is extremely sensitive when the clinical dose is taken as a basis of comparison. Since 0·5 to 1 c.c. are usually administered clinically (*e.g.*, in parturition), it will be seen that less than a thousandth of the clinical dose, or 0·0004 c.c. of the commercial (21 per cent.) extract, suffice to evoke the response.*

To obtain more crucial data it will be necessary to substitute for the crude method of injection *viâ* the dorsal lymph sac the intravenous or intraperitoneal operation. Considering the extreme technical facility of this method of testing the activity of pituitary preparations, the rapidity with which it can be carried out, the simplicity and cheapness of the materials employed and finally the probable identity—as will be seen later—of the melanophore and uterine stimulants, it is not premature to suggest in this place that the pituitary melanophore reaction merits the fullest consideration as an alternative to the very elaborate methods now employed for the standardisation of clinical preparations. It would be well moreover to explore the possibility of employing strips of frog skin as Hooker was successful in culturing the latter. It should then be possible to eliminate the factor of individual variation as a source of error. Spaeth emphasised this point in relation to the use of fish scales: the frog has the merit of being much more easily obtained and reared; and since excitement and mechanical stimulation ordinarily cause the frog's pigment cells to contract, everything favours the success of manipulation.

4. *Relation of the Melanophore Stimulant to other Pituitary Autocoids.*

It is now generally recognised that the diverse responses evoked by extracts of the posterior lobe of the pituitary gland are not due to a single autocoid.

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* 0·21 gr. fresh infundibular substance in 1 c.c.

By extraction of dried and powdered infundibulum with acidified water, treatment of the solution with colloidal ferric hydroxide and subsequent continuous extraction of the filtrate at reduced pressure with butyl alcohol, Dudley (1919) succeeded in separating a crystalline residue containing all the uterine stimulant (oxytocic principle) together with a portion of the pressor substance. The latter is again differentiated from the uterine stimulant by the fact that it is rapidly and completely destroyed by boiling with 0·5 per cent. HCl: on the other hand one-fifth of the uterine stimulant remains after half-an-hour of acid hydrolysis, and at the end of 6 hours a slight trace—less than 1/200ths persists. The rapid destruction of the pressor principle was shown by Abel and Nagayama (1920) whose results have been confirmed by Dale and Dudley (1921), and extended as indicated above. There are, therefore, at least two active principles in the extracts of the infundibulum; and the question thus arises of what relation exists between the melanophore stimulant of the infundibulum and the other pituitary autocoids.

The effect of continued acid hydrolysis was investigated as follows. A 0·5 per cent. solution of the commercial extract was made up in 0·5 per cent. HCl. This mixture was subjected to continuous boiling for 5 hours, a sample being removed at the end of 30 minutes. At the conclusion of the experiment a sample of the unboiled mixture, the portion which had been subjected to only half-an-hour's hydrolysis, and the residue were respectively neutralised with soda and diluted to a concentration approximately isotonic with Frog's Ringer. From each of the three solutions, A (unboiled), B (boiled half-an-hour), C (boiled 5 hours) 0·5 c.c. was injected into a pair of frogs whose pigment cells were fully contracted. The macroscopic and microscopic examination of the six animals at the conclusion of an hour revealed a marked contrast. The A and B pairs were dark and showed the typical pituitary reaction. The pair C remained pale. Microscopic preparations of the skin showed that in the C pair the melanophores were fully contracted, and in the A pair displayed extreme expansion. Those of the B pair were not so extremely expanded as those of the A pair. The result of the experiment indicates that pituitary extracts retain a considerable potency to induce melanophore response after half-an-hour's boiling with 0·5 per cent. HCl. Hence the melanophore stimulant is not identical with the pressor substance, and in its slow destruction by acid hydrolysis behaves in a manner identical with the uterine or oxytocic principle of infundibular extracts, as far as is demonstrable without extensive quantitative estimation of the potency of each sample.

5. *Relation of the Melanophore Stimulant to Histamine.*

The possibility that the melanophore stimulant present in infundibular extracts is identical with the oxytocic principle led to experiments on the effect of histamine, since Abel and Kubota (1919) have raised the question of the identity of histamine and the uterine stimulant. Dudley has since produced evidence that the effects of pituitary extract on plain muscle are not due to the presence of traces of histamine as these workers suggested, and more recently Dale and Dudley (1921) have conclusively shown that Abel and Kubota were wrong on this point. It can also be stated, with the utmost confidence, that the melanophore stimulant is likewise not identical with histamine; doses of the latter as considerable as 0·0036 grm. do *not* induce melanophore expansion in the frog.

That the two substances are not identical is shown further by the action of trypsin. Histamine is not acted upon by trypsin,* whereas Dale (1909) and Dudley (1919) have shown that both the commonly recognised pituitary autocoids are destroyed rapidly by trypsin. In this connection it may be noted that Schafer and Herring (1906) claimed that pepsin destroys the pressor activity of pituitary extract, leaving intact the diuretic principle; they denied that trypsin destroyed either. The following experiment indicates that pepsin does not affect the melanophore stimulant, but that the latter is rapidly destroyed by trypsin.

Six solutions were made up as follows:—

A. 0·5 per cent. infundibular extract in 0·2 per cent. HCl and 0·5 per cent. pepsin.

B. 0·5 per cent. infundibular extract in 0·2 per cent. HCl, without pepsin.

C. 0·2 per cent. HCl and 0·5 per cent. pepsin.

D. 0·5 per cent. infundibular extract in 0·5 per cent. saline trypsin.

E. 0·5 per cent. saline trypsin alone.

F. 0·5 per cent. infundibular extract in 0·5 per cent. boiled solution of trypsin.

At the conclusion of 2 hours' digestion at 34° C. A–C were neutralised, boiled, and diluted to a concentration approximately isotonic with Ringer. D–F were boiled. After cooling, 0·5 c.c. of each fluid was injected into a pair of frogs; and the 12 animals were examined 45 minutes afterwards, a strip of skin being removed for microscopic examination.

Of A–C both the A and B pairs showed macroscopically and microscopically the characteristic pituitary reaction. Of D–F, F alone showed expansion of the melanophores on being examined by macroscopic and microscopic methods.

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* Dudley (*op. cit.*).

The result of the experiment indicates that the melanophore stimulant of pituitary extracts is not destroyed by pepsin or HCl 0·2 per cent., but that it is completely and rapidly destroyed by trypsin. This is in accordance with Dale's work on the uterine and pressor principles.

As the foregoing experiments show, the melanophore stimulant, like the other pituitary autocoids, is not destroyed by boiling; it is immediately destroyed in the presence of a small quantity of H_2O_2. One other point deserves mention in this connection to correct a possibly erroneous interpretation of results previously published by one of the authors. It was stated (Huxley and Hogben, 1921) that pituitary feeding induces melanophore expansion in salamander larvæ. In view of the destruction of the melanophore stimulant by trypsin, it seems highly probable that the effect was not produced as in the case of thyroid feeding by absorption of the autocoid *viâ* the digestive system, but by traces in solution in the medium, acting through absorption by the skin.

6. *Mode of Action of the Melanophore Stimulant.*

The effect of injection of pituitary extracts on the intact animal might be interpreted in at least four different ways:—

(*a*) Local effect on the circulation (vasomotor).
(*b*) Central stimulation.
(*c*) Action on nerve-endings in melanophores.
(*d*) Direct action on the melanophores.

The first two are dismissed in view of the fact that, with appropriate preparations, pituitary extracts can be made to exert their characteristic reaction upon isolated skin. The second is also excluded by the possibility of producing the same reaction after destruction of both spinal cord and brain. To discriminate between (*c*) and (*d*), two methods may be adopted: first, the injection of pituitary extract after the complete degeneration of the nerve supply of the skin in a particular area; and, secondly, injection after paralysis of the nerve-endings which supply the melanophores by means of the usual drug series. The first is the more critical, and it is hoped to carry out this method later. The second is open to the objection that the evidence is based on the analogy of the operation of drugs on plain muscle. As far as the results of experiments on these lines permit legitimate inference, it would appear that the action of the infundibular melanophore stimulant is direct and independent of any nerve-endings.

The following re-agents were used for paralysis of different types of nerve-endings: cocaine (afferent), curare (spinal efferent), atropine (parasympathetic), and apocodeine (sympathetic). The use of cocaine was suggested by the possibility of an antidromic control. The subsequent action of pituitary extract was tested at varying intervals following doses of different magnitude, to make certain both as regards time of action and amount of drug administered, that the full effect of the latter would be exerted. In no case did the normal response fail to make its appearance. The indications are therefore that the pituitary melanophore stimulant acts directly on the melanophores of the frog and not on the nerve-endings, as is, indeed, fully consonant with what is at present known of the mode of action of the other pituitary autocoids.

Neither curare, atropine nor cocaine affect the melanophores of the frog in any way. While injection evoked its maximum effect in decerebrated frogs which had received just over the normal dose of curare adequate to produce general motor paralysis, it should be noted that very large doses of curare did prevent the melanophore response to pituitary injection before the circulation had actually ceased. Repeated tests, with a wide range of doses, showed that atropine does not cause the melanophores of the frog to expand as do those of the fish *Fundulus* when so treated (Spaeth). Laurens (1915) has also noted the inefficacy of atropine to induce expansion in the contracted melanophores of the Axolotl. The different mode of reaction of the dermal melanophores of Fishes and Amphibia towards both atropine and the pituitary autocoïd would suggest that the term "dermal melanophore" has been applied to more than one type of effector organ. It is intended to reserve, for fuller discussion at a later stage, the reactions of frog melanophores to drugs; but it may here be mentioned, in connection with the antagonism of atropine and pilocarpine in respectively paralysing and stimulating parasympathetic and secretory nerve-endings, that the latter re-agent does not induce expansion of the contracted melanophores in the frog. Were these structures innervated by the parasympathetic it would be expected that either one or the other would operate in this way. Apocodeine which, as Dixon (1904) has shown, effects general sympathetic paralysis, brings about a darkening of the skin, not sufficient, however, to mask the subsequent effect of pituitary administration. This is fully consonant with the reaction of the melanophores to adrenalin, and the direct evidence given by Hooker (1912) in regard to the relation of sympathetic stimulation to melanophore contraction.

Finally, the reaction studied in the experiments here recorded, leads to the possibility that the nervous system may not be such an all-important factor in the mechanism of "colour adaptation" as it has been customary to believe in the past. Laurens (1915) concludes from his experiments on the pigment responses of blinded and seeing axolotls, that in "colour adaptation" among the Amphibia the stimuli received by the retina and transmitted to the central nervous system are "indisputably" there "transformed and sent out along motor nerves to the pigment cells." That the retina exerts a controlling influence is admitted; that the stimuli it receives are transmitted to the central nervous system can hardly be questioned; but that the control of the pigment cells thereby is of a purely reflex character cannot be conceded till the endocrine factor has been eliminated. The existence of an endocrine substance capable of producing the reverse response to adrenalin,* raises the possibility that the central nervous system may exercise its control over the pigment cells partly at least through the secretory activity of the suprarenal and pituitary glands.

7. *Summary.*

1. Extracts of the posterior lobe of the pituitary gland have a specific effect on the melanophores of the frog causing them to undergo extreme expansion; an injection, equivalent to less than 1/1000th of the ordinary clinical dose, is adequate to produce a conspicuous darkening of the skin visible to the naked eye. The effect of pituitary extract on the dermal melanophores of the frog is thus the reverse of that which follows administration of adrenalin in the frog, and both adrenal and pituitary autocoids in Fundulus, if Spaeth's observations on the latter are correct.
2. The melanophore stimulant of pituitary extracts is only slowly destroyed by boiling with 0·5 per cent. HCl; it is hence not identical with the pressor principle, and in its slow destruction by acid hydrolysis agrees with the oxytocic or uterine principle.
3. That it is not identical with histamine is shown both by the inefficacy of this drug to induce expansion of the melanophores, and by the readiness with which it is destroyed by tryptic digestion; it is not destroyed in a corresponding manner by pepsin.
4. That the pituitary stimulant acts directly on the dermal melanophores rather than on the nerve endings is indicated by the failure of apocodeine, atropine, curare, and cocaine, to abolish the reaction when administered in doses which, on general grounds, would be regarded as sufficient to paralyse all nerve endings.

This research was carried out in Prof. McBride's laboratory; acknowledgment is made to Prof. McBride for his kindness in reading the MS., and to Dr. H. H. Dale for generous assistance with reference to the literature.

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* *Cf.* Introduction.

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*Relationships between Antiseptic Action and Chemical Constitution with special reference to Compounds of the Pyridine, Quinoline, Acridine and Phenazine Series.**

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BIOLOGICAL SECTION.

In the course of investigations on the antiseptic properties of certain basic benzene derivatives, it was shown that the methochloride of diaminoacridine was very highly antiseptic, and that, unlike other powerful antiseptics then known, its antibacterial activity was not reduced by the presence of blood serum (Browning, Gilmour, Gulbransen, Kennaway and Thornton, 2, 7). Diaminoacridine methochloride had been prepared by

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